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Polar compounds preclude mathematical lipid correction of carbon stable isotopes in deep-water sharks

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ABSTRACT

Lipids affect stable isotope values generated for marine fishes, however these effects remain poorly described for many extant shark taxa, especially deep-sea species. Here, we report the effects of lipid extraction (LE) on $\delta^{13}C$, $\delta^{15}N$, and C:N values of seven deep-sea sharks, generate novel mathematical normalizations for $\delta^{13}C$ based on the relationship between bulk and lipid extracted values ($\delta^{13}C_{Bulk}$ and $\delta^{13}C_{LE}$) and examine whether common normalized correction models provide a robust method for addressing lipid-biasing effects in two species, the Cuban dogfish (*Squalus cubensis*; $n = 20$), and Greenland shark (*Somniosus microcephalus*; $n = 24$). LE generally resulted in enrichment of ^{13}C and ^{15}N, but produced variable effects on C:N across all species. Novel mathematical normalizations for $\delta^{13}C$ were derived from the pooled shark community, and a single species-specific correction was generated for the Cuban dogfish, but could not be determined for the Greenland shark. Four common lipid correction models used for teleosts, failed to accurately predict $\delta^{13}C$ values statistically similar to $\delta^{13}C_{LE}$, in both Cuban dogfish and Greenland sharks, likely due to the confounding effects of lipids and urea on C:N. These observations suggest that chemical lipid extraction should be a mandatory procedure prior to interpreting stable isotope data for deep-sea sharks, at least for those species where lipid effects are large.

1. Introduction

Stable isotope analysis (SIA) of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) is a useful, low-cost approach to examine critical aspects of shark ecology (Hussey et al., 2012a; Shiffman et al., 2012). Thus far, SIA has been used to examine trophic interactions at inter- (e.g. Churchill et al., 2015a; Madigan et al., 2015) and intra-species scales (Estrada et al., 2006; Hussey et al., 2011), foraging breadth and energy flow (McCauley et al., 2012; Trueman et al., 2014), migration (Speed et al., 2012; Carlisle et al., 2012; Papastamatiou et al., 2015), and maternal provisioning (McMeans et al., 2009; Olin et al., 2011) in these taxa. Yet, accurate ecological interpretation of stable isotope data relies on confidence in a number of underpinning assumptions, for example assigning accurate trophic-step fractionation values (i.e. the relative isotopic enrichment of a predator relative to its prey), tissue turnover rates, and accounting for biasing effects of polar compounds (Logan and Lutcavage, 2010; Hussey et al., 2012a, 2012b; Shipley et al., 2017).

The confounding effects of polar compounds, namely lipids and urea/trimethylamine N-oxide (TMAO, herein referred to as urea), on isotope values of carbon and nitrogen are relatively well described for sharks (Hussey et al., 2011; Li et al., 2016; Carlisle et al., 2016). Lipids are ^{13}C-depleted relative to proteins, and osmolytes that facilitate cellular osmoconformation (Laxson et al., 2011), such as urea are ^{15}N-depleted relative to proteins, such that higher lipid and urea concentrations result in lower $\delta^{13}C$ and $\delta^{15}N$ values (Hussey et al., 2012b; Churchill et al., 2015b; Li et al., 2016). The combined effects of lipids and urea, if not accounted for, have the ability to confound accurate ecological interpretation of isotope data in sharks, and may result in biased conclusions regarding wider community dynamics (Kim and Koch, 2011; Hussey et al., 2012b; Li et al., 2016; Carlisle et al., 2016).

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Given these confounding factors, researchers must choose a suitable approach to offset the biasing effects of lipids, for which three commonly published approaches are preferred for sharks: 1. assume a negligible lipid content based on a low C:N ratio (< 3.4), and present bulk data (Pethybridge et al., 2012); 2. perform chemical extraction, which may additionally remove urea (Hussey et al., 2012b; Burgess and Bennett, 2017) or 3. mathematically correct for lipids using models generated for teleost fishes (Reum, 2011). Mathematically predicting 'lipid-free' $\delta^{13}C$ relies largely on the C:N of bulk tissue, in addition to the application of pre-determined lipid offsets (i.e. the isotopic depletion of lipid relative to protein, Sweeting et al., 2006), which vary between models e.g. Sweeting et al. (2006) (ca. −7.0‰), or McConnaughey and McRoy (1979) (ca. −6.0‰). As such, their application to normalize shark isotope data is unreliable, due to urea effects on $C:N_{Bulk}$ (Carlisle et al., 2016). Thus, the difficulty in generating broadly applicable mathematical normalizations for $\delta^{13}C$ and $\delta^{15}N$ in sharks has resulted in a consensus to chemically extract polar compounds prior to SIA (Kim and Koch, 2011; Li et al., 2016; Carlisle et al., 2016). Despite this argument, deep-sea species are largely unrepresented across the current literature base, highlighting a need to examine polar compound dynamics in these data-poor taxa.

In recent years, isotopic approaches have been applied to deep-water shark communities (Pethybridge et al., 2012; Churchill et al., 2015a; Shipley et al., 2017), to help drive novel management and conservation approaches. However, little consideration has been applied to the most appropriate techniques to account for lipid effects on $\delta^{13}C$ values in these taxa. Pethybridge et al. (2010) observed variable lipid content of deep-sea shark tissues, however those commonly run for SIA, e.g. white muscle, may be similarly low and comparable to pelagic and coastal species. However, as such analysis remains limited to a small number of species and locations, a need exists to further understand the biasing effects of lipids on deep-water shark tissue, and to provide a methodological proxy on which to base standardized sample preparation for future isotope studies. This is required because lipid-biasing effects in deep-sea taxa may be unique, as their physiologies and life-history characteristics diverge from that of coastal and pelagic species (Cotton and Grubbs, 2015).

Here we address the issue of whether commonly used mathematical correction models for $\delta^{13}C$ are appropriate for standardizing isotope bulk-tissue data for a number of data-poor deep-water sharks. First we quantify the changes (Δ) in $\delta^{13}C$, $\delta^{15}N$ and C:N following lipid extraction for this group. We then examine whether normalized correction models provide a robust method for addressing lipid-biasing effects, and generate novel mathematical normalizations based on the relationship between $\delta^{13}C_{Bulk}$ and $\delta^{13}C_{LE}$. This is critical given previous recommendations of lipid and urea removal prior to SIA (Hussey et al., 2012b; Li et al., 2016; Carlisle et al., 2016) but there is a lack of full evidence for this recommendation and a renewed call to address this issue in data-poor deep-water species.

2. Materials and methods

2.1. Tissue sampling and stable isotope analysis

Six species of shark: the Cuban dogfish (*Squalus cubensis*), gulper shark (*Centrophorus* spp.), big-eye sixgill shark (*Hexanchus nakamurai*), dusky smoothhound (*Mustelus canis-insularis*), sharpnose sevengill shark (*Heptranchias perlo*), and blotched catshark (*Scyliorhinus meadi*) were sampled from The Exuma Sound, The Bahamas from 2013 to 2014 (Table 1). Capture methods followed Brooks et al. (2015), and white muscle tissue was excised from the dorsal region, anterior to the first dorsal fin. Muscle samples were stored on ice before being frozen (− 20 °C) on return to the laboratory. Greenland sharks (*Somniosus microcephalus*) were sampled from Maxwell Bay, Lancaster Sound, The Canadian Arctic, in 2011 (Table 1). Individuals were caught using deep-water (~200 m) bottom-set long-lines (length: ~120 m), with 50 hooks baited with beluga meat and set for ~24 h. Sharks that were cannibalized on the line were sampled. White muscle tissue was excised immediately anterior to the first dorsal fin where possible or from posterior to the cranium or anterior to the caudal fin for severely cannibalized animals. Samples were immediately stored frozen (− 20 °C). Muscle tissues from all sharks were freeze-dried for > 72 h, homogenized and separated into paired bulk and lipid-extracted treatments (referred to as LE herein). Lipid extraction was undertaken using a 2:1 choroform:methanol approach following Sweeting et al. (2006).

For individuals captured in The Bahamas, stable isotope analysis of carbon and nitrogen was performed using a Sercon INTEGRA2 mass spectrometer (Sercon ltd, Cheshire, UK) at the University of Exeter, Penryn Campus (UK). All samples were run in duplicate, and internal analytical precision (standard error) was determined by running two alanine standards (n = 94, < 0.02) every 8 samples. Analytical precision between consecutive runs was determined using an in-house laboratory standard of blue antimora (*Antimora rostrata*), which were placed at the beginning and end of each run (n = 14, < 0.05). For Greenland sharks stable carbon and nitrogen isotope ratios were provided from a continuous flow isotope ratio mass spectrometer (IRMS, Finnigan MAT Deltaplus, Thermo Finnigan, San Jose, CA, USA) equipped with an elemental analyzer (Costech, Valencia, CA, USA) at the Chemical Tracers Laboratory - Great Lakes Institute for Environmental Research, University of Windsor, Canada. Precision, assessed by the standard deviation of replicate analyses of four standards (NIST1577c, internal lab standard (tilapia muscle), USGS 40 and urea (n = 13 for all), measured ≤ 0.17‰ for $\delta^{15}N$ and ≤ 0.16‰ for $\delta^{13}C$ for all standards. The accuracy, based on the certified values of USGS 40 (n = 13 for $\delta^{13}C$) and urea IVA33802174 (n = 13 for $\delta^{15}N$) analyzed throughout runs and not used to normalize samples showed a difference of −0.16‰ for $\delta^{15}N$ and −0.08‰ for $\delta^{13}C$ from the certified value. Instrumentation accuracy checked throughout the period of time that these samples were analyzed was based on NIST standards 8573 and 8574 for $\delta^{15}N$ and 8542,8573, 8574 for $\delta^{13}C$ (n = 18 for all). The mean difference from the certified values was −0.14, and 0.04‰ for $\delta^{15}N$ and −0.05, −0.03 and −0.05‰ for $\delta^{13}C$ respectively.

2.2. Statistical analysis

To assess the effects of LE on isotope values, differences in $\delta^{13}C$, $\delta^{15}N$ and C:N between LE and bulk tissue were determined using Student's t-tests or Wilcoxon signed ranks test based on Shapiro-Wilks normality (α = 0.05). For Cuban dogfish (*Squalus cubensis*; n = 20) and Greenland sharks (*Somniosus microcephalus*; n = 24) least squares linear regression was used to assess whether $\Delta\delta^{13}C$ could be predicted based on the C:N of bulk tissue; this would provide further evidence of whether lipid or urea effects were more responsible for changes in C:N in these species. Bulk and LE data are presented for five additional deep-water species to show overall isotopic trends between treatments in support of the above two species, but were not rigorously tested due to small sample sizes ($n \geq 6$). Although the sample size for *Centrophorus* spp. met the criteria for analysis, they were excluded as on-going taxonomic revision precluded positive species ID during sampling (see White et al., 2013; Verissimo et al., 2014; Brooks et al., 2015).

Mathematical normalization of $\delta^{13}C$ was established for species with a sample size > 20 through a linear function based on LE and bulk $\delta^{13}C$ following Li et al. (2016):

$$\delta^{13}C_{LE} = a_1 \times \delta^{13}C + b_1 \quad (1)$$

where a_1 is the slope and b_1 is the intercept.

To test the performance of mathematical lipid correction models on $\delta^{13}C$, we considered four common mathematical correction approaches. The first correction requires the calculation of the relative proportion of